Letter to the Editor in Response to Zhou et al.

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In their recent publication, Zhou et al[1] describe a concentration-dependent increase in rate of mutation in a modified in vitro Chinese hamster ovary cell HPRT assay with N-hydroxycytidine (NHC). NHC is the parent nucleoside of the 5'-isopropylester prodrug molnupiravir (MOV). In contrast, we have conducted a more comprehensive series of in vitro and in vivo genotoxicity studies which, based on the totality of the data, demonstrate a low risk for genotoxicity with MOV in clinical use. We review these studies, as well as potential concerns with the methods used by Zhou et al.

While MOV and/or NHC have demonstrated the ability to induce mutations under specific in vitro culture conditions (including Ames and modified HPRT assays), extensive study of MOV in *in vivo* whole animal mutagenicity assays provides strong evidence of lack of *in vivo* relevance. Potential reasons for lack of translation of *in vitro* findings to *in vivo* mammalian systems may involve differences in metabolism, pharmacokinetics, exposure, replication, and DNA repair processes within a whole animal model compared with *in vitro* test conditions. It is well-recognized that studies in appropriate *in vivo* models are needed to establish the biological significance and clinical risk of *in vitro* assay findings. As such, we conducted two distinct rodent

mutagenicity *in vivo* models which are recognized as robust tools for evaluating mutagenicity *in vivo*, and for assessing human risk for mutagenicity [Pig-a mutagenicity assay and Big Blue® (cII Locus) transgenic rodent assay] [2, 3]. In the Pig-a mutagenicity assay and Big Blue® (cII Locus) transgenic rodent assay, the impact of MOV treatment on mutation rates was not differentiable from mutation rates observed in untreated historical control animals. These *in vivo* mutation assays evaluated MOV at doses and durations significantly greater than those being used in the clinic. MOV was also negative for induction of chromosomal damage in *in vitro* micronucleus (with and without metabolic activation) and *in vivo* rat micronucleus assays. Thus, based on the totality of genotoxicity data (including two distinct in vivo rodent mutagenicity models in which MOV did not demonstrate evidence of mutagenicity or genotoxicity *in vivo*), MOV is considered of low risk for genotoxicity in clinical use.

It is important to note that the assay conditions used for the *in vitro* HPRT assay by Zhou et al were significantly different from standard protocols conducted under regulatory test guidelines [4]. The following provides a critical analysis of assay methods described in the supplemental materials from Zhou et al, highlighting several features that make interpretation of the results and comparison with existing published HPRT data problematic:

- The cells were exposed continuously to NHC for a total of 32 days, substantially longer than the 3-6-hour exposure duration typically used per established guidelines [4]. Historical control data (used to determine performance of the assay in the laboratory with different positive and negative controls, and to establish acceptable background mutant frequency ranges in untreated cells) are not provided by the authors [5].

- While NHC was shown to be toxic to CHO-K1 cells, when exposed at 10 µM for 5 days (shown in supplementary figure 4 in Zhou et al), cytotoxicity was not assessed at the end of the 32-day continuous exposure to NHC at ≤3 µM. This step is needed to assess whether there was a reduction in relative survival of the treated cells compared with the control to help differentiate direct test article-related mutagenicity versus mutations that may occur due to DNA damage induced under cytotoxic conditions [6, 7].

- The mutation results provided by Zhou et al were reported as total mutant colonies rather than mutant frequency [1], which does not allow for comparison of negative and positive control data to publicly available literature.

- The rationale for the NHC concentrations used in the assay (or concurrent control compounds) was not provided. To avoid potential false positive results, the highest concentration tested should avoid producing precipitation in the culture media, marked changes in pH or osmolality, or excessive cytotoxicity [4].

- Information regarding origin and purity of the NHC material used were not provided, and it is uncertain whether stability or impurity characterization of the material was conducted.

Given the state of the current COVID-19 pandemic and the repeated and accelerating emergence of highly pathogenic coronaviruses, the development of potent antivirals with activity against SARS-CoV-2 and other coronaviruses is urgently needed. Our comprehensive safety evaluation coupled with the preclinical antiviral efficacy and clinical experience to date support the ongoing studies of molnupiravir in patients, including those most likely to benefit from early intervention.

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